Butylated hydroxytoluene (BHT) – Determination of 3,5 di-tert-butyl-4-hydroxybenzoic acid (BHT acid) in urine by LC- MS/MS. Biomonitoring Method – Translation of the German version from 2020

Hartwig, Andrea ; MAK Commission ; et al ; Arand, Michael

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Keywords

butylated hydroxytoluene, BHT, 2,6-di-*tert*-butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxytoluene, urine, BHT acid, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, biomonitoring, LC-MS/MS

W. Gries[1]
K. Küpper[1]
C. Schmidtkunz[1]
G. Leng[1]
G. Scherer[2]
D. Krnac[2]
T. Göen[3,*]
A. Hartwig[4,*]
MAK Commission[5,*]

[1] *Method development, Currenta GmbH & Co. OHG, CUR-SEL-SER-GS-BLM, Institut für Biomonitoring, 51368 Leverkusen, Germany*
[2] *External verification, ABF – Analytisch-biologisches Forschungslabor GmbH, Goethestraße 20, 80336 München, Germany*
[3] *Chair of the working group "Analyses in Biological Materials", Deutsche Forschungsgemeinschaft, Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, Friedrich-Alexander University Erlangen-Nürnberg, Henkestraße 9–11, 91054 Erlangen, Germany*
[4] *Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany*
[5] *Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany*

* *E-Mail: T. Göen (thomas.goeen@fau.de), A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)*

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area verified the presented biomonitoring method.

The method described hereinafter permits the selective determination of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT acid) as a metabolite of butylated hydroxytoluene (BHT) in urine. After adding a $^{13}C_6$-ring labelled internal standard (ISTD), the samples are enzymatically hydrolysed to release the free BHT acid from glucuronidated compounds. Using two-dimensional liquid chromatography the samples are concentrated and separated from matrix components and analysed using tandem mass spectrometry. Calibration is done using calibration standards prepared in water and processed in the same way as the samples to be analysed.

1 Characteristics of the method

Matrix Urine

Analytical principle LC-MS/MS

Parameter and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
Butylated hydroxytoluene (BHT)	128-37-0	3,5-Di-*tert*-butyl-4-hydroxybenzoic acid (BHT acid)	1421-49-4

Reliability data

3,5-Di-*tert*-butyl-4-hydroxybenzoic acid (BHT acid)

Within-day precision:	Standard deviation (rel.)	s_w = 4.0%, 5.1%, 4.5% or 6.0%
	Prognostic range	u = 8.9%, 11.4%, 10.0% or 13.5%
	at a spiked concentration of 0.1 µg, 1.0 µg, 10.0 µg or 100 µg BHT acid per litre water and where n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 9.7%, 8.1%, 11.0% or 10.0%
	Prognostic range	u = 21.8%, 18.2%, 24.8% or 22.5%
	at a spiked concentration of 0.1 µg, 1.0 µg, 10.0 µg or 100 µg BHT acid per litre water and where n = 10 determinations	
Accuracy:	Recovery rate (rel.)	r = 101%, 103%, 101% or 102%
	at a spiked concentration of 0.1 µg, 1.0 µg, 10.0 µg or 100 µg BHT acid per litre water and where n = 10 determinations	
Detection limit:	0.06 µg BHT acid per litre water	
Quantitation limit:	0.2 µg BHT acid per litre water	

2 General information on BHT

Butylated hydroxytoluene (BHT) (2,6-di-*tert*-butyl-*p*-cresol, 3,5-di-*tert*-butyl-4-hydroxytoluene) was patented in 1947 and initially used as a stabiliser in the petroleum and adhesives industries. Due to its antioxidant properties, its application range was extended back in the 1950s to include the stabilisation of foodstuffs and cosmetics (Witschi et al. 1989). Today it is one of the most prevalent synthetic antioxidants (Nieva-Echevarria et al. 2015), the antioxidant properties of which are attributable to the radical scavenging, sterically hindered phenol group.

Accordingly, the range of applications in the food sector is wide: it is added as an antioxidant (E 321) in particular to baking mixtures, nuts, packet soups, chewing gums as well as fats and oils. Regulation (EC) No. 1333/2008 (EU 2008) sets maximum contents for this additive in various foodstuffs. Other applications include the addition in pharmaceuticals (ointments, gelatine capsules), cosmetics (shower gels, soaps), plastics and rubber products as well as paints and varnishes.

BHT is industrially produced by the reaction of *p*-cresol with 2-methylpropene catalysed by sulphuric acid. The global production volume in 2000 was approximately 62,000 t/a (OECD 2002).

BHT is a white solid consisting of colourless crystals and has a mild phenolic odour. Its melting point is 70 °C and it is almost insoluble in water (0.76 mg/l). BHT is marketed under various trade names, including Vulkanox-BHT, Ionol, Paranox 441, Impruvol or Antioxidant 4K.

At the workplace inhalation is the major route of exposure to BHT, but dermal absorption is also possible, although after dermal application only 4% of the applied amount penetrates the skin, indicating that systemic bioavailability is rather low (Henschler 1986).

Several metabolic pathways and numerous metabolites are known for BHT. Metabolism studies of BHT in rats, mice and rabbits have shown that chemical reactions during metabolism can take place in three positions: on the ring, on the methyl group as well as on the *tert*-butyl groups. The primary metabolic pathway in rodents and humans leads to BHT acid by stepwise oxidation of the 4-methyl group via BHT alcohol and BHT aldehyde (see Figure 1) (Henschler 1986; Nieva-Echevarria et al. 2015). The few available data on the metabolism of BHT in humans show that up to 5% of the resorbed BHT dose is excreted in urine as BHT acid, most of which is glucuronidated (Daniel et al. 1968; Verhagen et al. 1989; Wiebe et al. 1978).

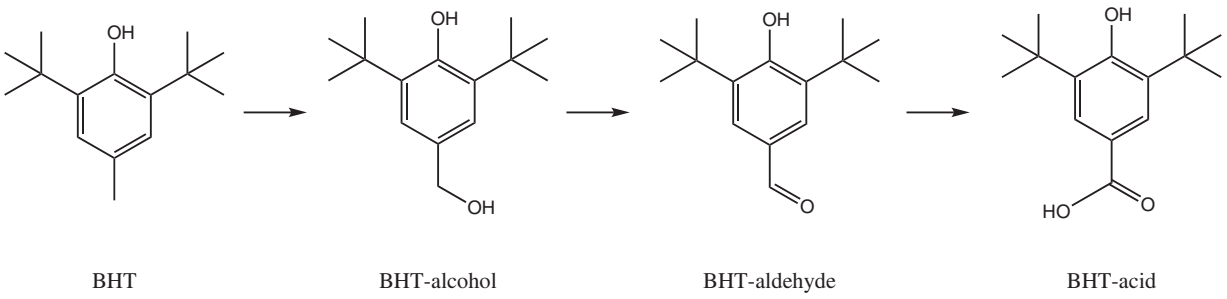

Fig. 1 Primary metabolic pathway of BHT to BHT acid (Henschler 1986)

Elimination takes place in two phases: in the α-phase, elimination is rapid and takes place predominantly via the urine. In the β-phase, the elimination half-life is approximately 10 days as a result of an extensive enterohepatic circulation of the metabolites or their accumulation in adipose tissue. In humans, BHT and its metabolites are excreted primarily in the urine, while in rats they are also eliminated via the faeces (Greim 2007; Lanigan and Yamarik 2002).

BHT was classified by the Commission as a Category 4 carcinogen. The MAK value was set at 10 mg BHT/m^3 E (DFG 2019; Hartwig 2012). Since absorption through the skin does not contribute significantly to the internal load, BHT was not designated with an "H". It has not been designated with "Sh" or "Sa" either. EFSA derived an ADI level (Acceptable Daily Intake) of 0.25 mg/kg body weight per day (EFSA 2012). Reference or limit values in biological material are not available. For further details on the toxicological evaluation of BHT, please refer to the respective MAK value documentations of the Commission (Greim 2007; Hartwig 2012; Henschler 1986).

Due to the widespread use of BHT in many areas of life, a potential exposure of the general population can be assumed. Table 1 shows data on the background exposure of the general population from several studies.

Tab. 1 Data on the background exposure of the general population to BHT, specifying the BHT acid excretion in urine

Country	Number of samples (> LOQ)	BHT acid [µg/l]		Reference
		Mean value (median)	Range	
Japan	24 (91.7%)	5.49 (3.86)	< LOQ–24.4	Wang and Kannan 2019
India	36 (94.3%)	5.15 (2.24)	< LOQ–24.0	
China	53 (80.4%)	0.56 (0.26)	< LOQ–4.98	
Saudi Arabia	9 (100%)	1.46 (0.44)	0.11–5.63	
U.S.	23 (95.7%)	7.44 (1.78)	< LOQ–46.0	
Germany	40 (82.5%)	1.28 (0.83)	< LOQ–7.55	Leng and Gries 2017
Germany	10 (100%)	8.66 (3.75)	0.7–35.4	data obtained during external method verification
Germany	329 (98%)	1.70 (1.06)	< LOQ–18.1	Schmidtkunz et al. 2020
Germany	22 (91%)	n. s. (0.91)	< LOQ–12.7	Göen et al. 2006

LOQ: limit of quantitation; n. s.: not specified

3 General principles

The method described hereinafter permits the selective determination of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHT acid) as a metabolite of butylated hydroxytoluene (BHT) in urine. After adding a $^{13}C_6$-ring labelled internal standard (ISTD), the samples are enzymatically hydrolysed to release the free BHT acid from glucuronidated compounds. Using two-dimensional liquid chromatography the samples are concentrated and separated from matrix components and analysed using tandem mass spectrometry. Calibration is done using calibration standards prepared in water and processed in the same way as the samples to be analysed.

4 Equipment, chemicals and solutions

4.1 Equipment

- UPLC-MS/MS system: Waters Acquity UPLC with 2D-LC option coupled with Waters Xevo-TQS tandem mass spectrometer (Waters GmbH, Eschborn, Germany)
- LC column: Zorbax RRHD Eclipse Plus C8 1.8 µm, 2.1 mm × 100 mm (Agilent Technologies Deutschland GmbH, Waldbronn, Germany, No. 959758-906)
- Pre-concentration column: XBridge BEH C8 Direct Connect HP 10 µm, 2.1 mm × 30 mm (Waters GmbH, Eschborn, Germany, No. 186005233)
- Trap column, e.g. Oasis HLB Direct Connect HP 20 µm, 2.1 mm × 30 mm (Waters GmbH, Eschborn, Germany, No. 186005231)
- Laboratory shaker (e.g. IKA Werke GmbH & Co. KG, Staufen, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Incubator (e.g. Memmert GmbH & Co. KG, Schwabach, Germany)
- Various volumetric flasks and beakers (e.g. BRAND GMBH + CO KG, Wertheim, Germany)

- Microlitre pipettes, variably adjustable from 10 µl to 100 µl as well as from 100 µl to 1000 µl (e.g. Eppendorf AG, Hamburg, Germany)
- Multipette (e.g. Eppendorf AG, Hamburg, Germany)
- 2 ml vials and crimp caps (e.g. Waters GmbH, Eschborn, Germany, No. 186000327C)
- pH meter (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Universal pH test paper, pH 0–11 (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- Urine containers (e.g. Sarstedt AG & Co. KG, Nürnbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be at least p.a. grade.

- Acetonitrile, hypergrade for LC-MS (e.g. Merck KGaA, Darmstadt, Germany, No. 1.00029)
- Ultrapure water, CHROMASOLV™ (e.g. Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany, No. 39253)
- *β*-Glucuronidase (e.g. Roche Diagnostics Deutschland GmbH, Mannheim, Germany, No. 03707598001)
- Glacial acetic acid, Suprapur® (e.g. Merck KGaA, Darmstadt, Germany, No. 1.00066)
- Formic acid (e.g. Merck KGaA, Darmstadt, Germany, No. 533002)
- Ammonium acetate (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 73594)
- 3,5-Di-*tert*-butyl-4-hydroxybenzoic acid (BHT acid), 98% (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 143472)
- 3,5-Di-*tert*-butyl-4-hydroxybenzoic acid-$^{13}C_6$ (ring substituted) ($^{13}C_6$-BHT acid), 97% (custom synthesis, Institut für Dünnschichttechnologie und Mikrosensorik e. V., Teltow, Germany)

4.3 Solutions

- Ammonium acetate buffer (1 mol/l)

  Exactly 38.5 g ammonium acetate are weighed into a 400 ml beaker and dissolved in approximately 250 ml ultrapure water. After the pH has been adjusted to 6.5 (pH meter) with glacial acetic acid, the solution is quantitatively transferred to a 500 ml volumetric flask. The flask is then made up to the mark with ultrapure water.

- Mobile phase for UPLC (mobile phase A)

  1 ml formic acid is pipetted into a 1000 ml volumetric flask. The flask is then filled up to the mark with ultrapure water and the solution well mixed.

- Mobile phase for UPLC (mobile phase B)

  1 ml formic acid is pipetted into a 1000 ml volumetric flask. The flask is then filled up to the mark with acetonitrile and the solution well mixed.

The solutions are stable at 4 °C for at least six months.

4.4 Internal standard (ISTD)

- ISTD stock solution I (1000 mg/l)

  10 mg of the $^{13}C_6$-BHT acid are weighed exactly into a 10 ml volumetric flask and dissolved in acetonitrile. The flask is then made up to the mark with acetonitrile.

- ISTD stock solution II (10 mg/l)

  100 µl of the ISTD stock solution I are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- ISTD spiking solution (1 mg/l)

  1 ml of the ISTD stock solution II is pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

The ISTD solutions are stable for at least three months when stored in the refrigerator at 4 °C.

4.5 Calibration standards

- Stock solution (1000 mg/l)

  10 mg BHT acid are weighed into a 10 ml volumetric flask and dissolved in acetonitrile. The flask is then made up to the mark with acetonitrile.

- Spiking solution I (10 mg/l)

  100 µl of the stock solution are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- Spiking solution II (1 mg/l)

  1 ml of spiking solution I is pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- Spiking solution III (100 µg/l)

  100 µl of spiking solution I are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- Spiking solution IV (10 µg/l)

  100 µl of spiking solution II are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- Spiking solution V (1 µg/l)

  1 ml of spiking solution IV is pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

The stock solutions and spiking solutions can be stored at 4 °C for at least three months without analyte loss.

Calibration standards in the concentration range between 0.01 µg/l and 200 µg/l are prepared in ultrapure water according to the pipetting scheme shown in Table 2. Ultrapure water is included as a blank value.

Tab. 2 Pipetting scheme for the preparation of calibration standards used to determine BHT acid in urine

Calibration standard	Spiking solution	Volume of the spiking solution [µl]	Volume of the water used [µl]	Concentration of the calibration standard [µg/l]
1	–	–	500	0
2	V	5	495	0.01
3	V	10	490	0.02
4	V	25	475	0.05
5	IV	5	495	0.1
6	IV	10	490	0.2
7	IV	25	475	0.5
8	III	5	495	1.0
9	III	10	490	2.0
10	III	25	475	5.0
11	II	5	495	10.0
12	II	10	490	20.0
13	II	25	475	50.0
14	I	5	495	100
15	I	10	490	200

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in suitable urine collection containers and stored at −20 ℃ until analysis.

5.2 Sample preparation

Prior to analysis, the samples are thawed at room temperature and mixed thoroughly. For sample preparation, 0.5 ml of the urine sample are transferred into a 2 ml crimp top vial and 5 µl of the ISTD spiking solution are added. Afterwards, 1 ml of the ammonium acetate buffer is added. The sample is thoroughly mixed on a laboratory shaker. For hydrolysis, 5 µl of β-glucuronidase are added to the sample, the vial is sealed and the solution is thoroughly mixed. The sample is incubated for three hours in the incubator at 37 ℃. After cooling to room temperature, the sample is directly injected into the UPLC-MS/MS system for analysis.

6 Operational parameters

Analysis was performed using a UPLC system with two gradient pumps coupled to a tandem mass spectrometer.

6.1 Ultra performance liquid chromatography (UPLC)

Analytical column:	Zorbax RRHD Eclipse Plus C8 1.8 µm, 2.1 mm × 100 mm
Pre-concentration column:	XBridge BEH C8 Direct Connect HP 10 µm, 2.1 mm × 30 mm
Mobile phase:	Mobile phase A: ultrapure water with 0.1% formic acid Mobile phase B: acetonitrile with 0.1% formic acid
Column temperature:	40 °C
Injection volume:	25 µl
Column switching program:	0–0.5 min: valve 1 – position 1; valve 2 – position 1 0.5–3.5 min: valve 1 – position 2; valve 2 – position 1 3.5–10 min: valve 1 – position 1; valve 2 – position 1 (cf. Figure 2 in the Appendix)
Gradient program:	see Tables 3 and 4

Tab. 3 Program of gradient pump 1

Time [min]	Mobile phase A [Vol.-%]	Mobile phase B [Vol.-%]	Flow rate [ml/min]
0.0	90	10	0.8
1.0	90	10	0.8
1.5	0	100	0.8
3.0	0	100	0.8
3.5	90	10	0.8
10	90	10	0.8

Tab. 4 Program of gradient pump 2

Time [min]	Mobile phase A [Vol.-%]	Mobile phase B [Vol.-%]	Flow rate [ml/min]
0.0	90	10	0.3
1.0	90	10	0.3
7.0	5	95	0.3
9.0	5	95	0.3
9.5	90	10	0.3
10	90	10	0.3

6.2 Tandem mass spectrometry

Ionisation type:	positive electrospray ionisation (ESI+)
Source temperature:	150 °C
Desolvation temperature:	400 °C
Cone gas flow:	150 l/h
Desolvation gas flow:	700 l/h
Collision gas flow:	0.15 ml/h
Nebuliser gas flow:	6.0 bar
Parameter-specific settings:	see Table 5

Tab. 5 Parameter-specific settings for the analyte and the internal standard

Analyte	Retention time [min]	Ion trace [*m/z*]		Cone voltage [V]	Collision energy [V]
		Precursor ion	Daughter ion		
BHT acid	6.11	251.3	195.1	33.0	20.0
			139.0[a)]	33.0	20.0
			57.1[a)]	33.0	16.0
$^{13}C_6$-BHT acid	6.11	257.3	201.1[a)]	47.0	22.0
			145.0[a)]	47.0	20.0
			57.1	47.0	16.0

[a)] Qualifier

All settings are instrument-specific and must be adjusted individually by the user. The parameters specified above are therefore intended as a rough guide only. All other parameters have to be optimised in accordance with the manufacturer's specifications.

7 Analytical determination

25 µl each of the samples prepared as described in Section 5 are injected into the UPLC-MS/MS system. The analyte is enriched on the pre-column and then backflushed to the analytical column for analytical separation. Identification of the analyte is based on the retention time and the specific ion transitions. The retention times of the analyte and of the internal standard as well as the recorded ion transitions are listed in Table 5. The qualifier transitions are only used to confirm the identity of the analyte. Depending on matrix interferences, it may be advisable in some cases to quantify the analyte using one of the qualifiers instead of the actual quantifier. In such cases, the calibration graph must be created accordingly.

The retention times given in Table 5 are intended as a rough guide only. Users must ensure proper separation performance of the UPLC column used influencing the resulting retention behaviour of the analyte. Figures 3 and 4 (in the Appendix) show, as an example, chromatograms of a native urine sample and of a spiked water sample (calibration standard).

8 Calibration

The calibration standards described in Section 4.5 are prepared and processed in the same way as the samples (cf. Section 5.2) and analysed using LC-MS/MS (cf. Section 6). Calibration graphs are obtained by plotting the quotients of the peak areas of the analyte and of the $^{13}C_6$-labelled internal standard against the spiked concentration of the respective calibration standards. A quadratic calibration graph is obtained in the concentration range from the detection limit to 200 µg/l. Three reagent blanks (ultrapure water instead of the urine sample) and three quality control samples (see Section 10) are included in each analytical run.

Figure 5 (in the Appendix) shows an example of a calibration graph of the analyte prepared in water. Calibration is performed in water instead of urine due to the ubiquitous background levels of BHT acid in human urine.

9 Calculation of the analytical results

The determined peak areas of the BHT acid are divided by the peak areas of the $^{13}C_6$-labelled internal standard. The quotients thus obtained are entered in the calibration function established according to Section 8 to give the analyte concentration in µg/l. Any reagent blank values have to be subtracted from the analytical results. The analyte concentration was calculated using the Waters MassLynx 4.1 software.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) and in a general chapter of the MAK Collection for Occupational Health and Safety (Bader et al. 2010; Bundesärztekammer 2014). To check precision, at least three quality control samples with known analyte concentrations are analysed within each analytical run. As material for quality control is not commercially available, it must be prepared in the laboratory. To this end, spiking solutions of the analyte are added to ultrapure water, so that the concentrations of the control materials are within the relevant concentration range (e.g. 1 µg/l, 10 µg/l and 100 µg/l). For this purpose, the spiking solutions are prepared separately of those used for calibration (separate weighing procedure). Aliquots of these samples are stored at −20 °C and included in each analytical run as quality control samples. The nominal values and the tolerance ranges of the quality control materials are determined in a pre-analytical period (one analysis of the control materials each on 10 different days) (Bader et al. 2010).

At the same time, at least three reagent blanks are included in each analytical run to identify potential interferences caused by the chemicals used (cf. Section 11.5).

11 Evaluation of the method

The reliability of the method was verified by comprehensive validation as well as by implementation and validation of the method in a second, independent laboratory.

11.1 Precision

Within-day precision was determined in both water and urine. To this end, ultrapure water was spiked with BHT acid at concentration levels of 0.1 µg/l, 1.0 µg/l, 10 µg/l and 100 µg/l, respectively, and analysed ten times in a row. In addition, pooled urine was spiked with BHT acid at concentration levels of 1.0 µg/l and 10 µg/l, respectively, and analysed ten times in a row. The spiked samples were prepared and analysed as described in Section 5.2 and Section 6. Moreover, an unspiked native urine sample was processed nine times and analysed.

The thus obtained within-day precision data are presented in Table 6.

Tab. 6 Within-day precision for the determination of BHT acid (n = 9 and n = 10, respectively)

Matrix	Spiked level [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Water	0.1	4.0	8.9
	1.0	5.1	11.4
	10.0	4.5	10.0
	100	6.0	13.5
Pooled urine	1.0	11.4	25.8
	10.0	11.0	24.9
Unspiked urine sample	–[a)]	6.6	15.2

[a)] determined mean native concentration: 26.8 µg/l

To determine day-to-day precision, water was spiked and analysed ten times over a period of 2.5 days. The thus obtained day-to-day precision data are presented in Table 7.

Tab. 7 Day-to-day precision for the determination of BHT acid (n = 10)

Matrix	Spiked level [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Water	0.1	9.7	21.8
	1.0	8.1	18.2
	10.0	11.0	24.8
	100	10.0	22.5

11.2 Accuracy

The accuracy of the method was determined on the basis of the within-day precision data and the day-to-day precision data. In addition, ten individual urine samples were spiked with 1.0 and 10 µg/l BHT acid each, respectively, to determine the impact of different urine matrices. The relative recovery rates thus obtained are presented in Tables 8 and 9.

Tab. 8 Relative recovery rates for the determination of BHT acid, derived from the within-day precision data (n = 10) or from the analysis of spiked individual urine samples (n = 10)

Matrix	Spiked level [µg/l]	Recovery (rel.) r [%]	Range [%]
Water	0.1	101	95–108
	1.0	103	95–109
	10.0	101	93–105
	100	102	93–109
Pooled urine	1.0	95	80–111
	10.0	87	73–99
Individual urine samples	1.0	97	78–119
	10.0	95	85–105

Tab. 9 Relative recovery rates for the determination of BHT acid, derived from the day-to-day precision data (n = 10)

Matrix	Spiked level [µg/l]	Recovery (rel.) *r* [%]	Range [%]
Water	0.1	97	85–115
	1.0	101	88–112
	10.0	96	82–117
	100	98	88–113

11.3 Hydrolysis

In order to assess the need for hydrolysis, 17 native urine samples from individuals who could have come into contact with BHT at their workplace were processed and analysed with and without hydrolysis. The level of free BHT acid (without hydrolysis) and total BHT acid (free plus conjugated BHT acid, with hydrolysis) was determined. The results obtained are shown in Table 10.

Tab. 10 Determination of BHT acid in urine with or without enzymatic hydrolysis (n = 17)

Hydrolysis	BHT acid [µg/l]		> LOQ [%]
	Median (mean value)	Range	
without hydrolysis	0.52 (2.07)	< 0.2–12.1	82.4
enzymatic hydrolysis (*β*-glucuronidase)	4.11 (8.44)	0.9–32.5	100

In order to compare enzymatic hydrolysis with acidic hydrolysis, urine samples of 22 individuals who were occupationally exposed to BHT were analysed. The results are presented in Table 11.

Tab. 11 Determination of BHT acid in urine using enzymatic or acidic hydrolysis (n = 22)

Hydrolysis	BHT acid [µg/l]		> LOQ [%]
	Median (mean value)	Range	
enzymatic hydrolysis (*β*-glucuronidase)	4.55 (6.47)	0.7–26.6	100
acidic hydrolysis (hydrochloric acid)	3.84 (6.05)	0.5–26.7	100

Moreover, five of the high-exposure samples from this group were selected and compared regarding their results following hydrolysis with acid or with two different enzymes (glucuronidase or glucuronidase/arylsulfatase). The results are presented in Table 12.

Tab. 12 Determination of BHT acid in urine using two different enzymes or acidic hydrolysis (n = 5)

Hydrolysis	BHT acid [µg/l]				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
enzymatic hydrolysis (*β*-glucuronidase/arylsulfatase H. pomatia)	26.6	n. s.	10.5	24.3	11.4
enzymatic hydrolysis (*β*-glucuronidase E. coli)	24.4	5.03	10.7	20.3	13.1
acidic hydrolysis (hydrochloric acid)	19.6	4.48	9.06	26.7	14.4

n. s. = not specified (due to matrix interferences of the internal standard)

In order to determine the reproducibility of the enzymatic hydrolysis, a native urine sample was processed ten times and then analysed. The obtained data from nine measurements are shown in Table 13.

Tab. 13 Reproducibility of enzymatic hydrolysis when determining BHT acid in urine (n = 9)

Hydrolysis	Mean value [µg/l]	Range [µg/l]	Standard deviation (rel.) s_w [%]
enzymatic hydrolysis (β-glucuronidase)	26.8	23.9–29.6	6.6

11.4 Limit of detection and limit of quantitation

The detection limit and quantitation limit were determined according to DIN 32645 (DIN 2008) using the six lowest points of a calibration curve prepared in water. The limit of detection and quantitation calculated on the basis of the method standard deviation s_{x0} (LOD = 4 × s_{x0}) are shown in Table 14.

Tab. 14 Limit of detection and quantitation for the determination of BHT acid

Analyte	Matrix	Detection limit [µg/l]	Quantitation limit [µg/l]
BHT acid	Water	0.06	0.2

11.5 Sources of error

The objective of the method development was to reach a detection limit of at least 0.1 µg/l. This proved to be very ambitious as a distinct background level of BHT acid was observed in all urine samples analysed. Due to this background level, calibration in urine was not feasible. Therefore, the calibration standards were prepared in water, thus resolving the problem of background levels. Despite calibration in water, a constant blank value of approximately 0.3 µg/l was still detected. The ammonium acetate used, which contained traces of BHT acid despite its p.a. specification, was identified as the main source of this blank value. The blank could be reduced to approximately 0.12 µg/l by using ultrapure ammonium acetate for mass spectrometry. The deionised water (Milli-Q water) initially used was also considered as a possible source of BHT acid, as microbial degradation of BHT from the filter material could not be ruled out. The water was thus replaced by ultrapure LC-MS grade water and, at the same time, a trap column was installed upstream of the analytical column for each LC pump, respectively. These measures helped to reduce the blank to approximately 0.05 µg/l.

During method development, the need of hydrolysis in order to release the BHT acid from its conjugates was confirmed (see Section 11.3). Subsequently, various hydrolysis options were tested. It was observed that no significant difference in the release of BHT acid could be detected when using glucuronidase and glucuronidase/arylsulfatase compared to acidic hydrolysis. However, outliers were repeatedly detected in spiked urine samples following acidic hydrolysis (probably caused by quenching). And, after enzymatic hydrolysis with glucuronidase/arylsulfatase, elevated background levels of BHT acid were found, which was not the case after hydrolysis using glucuronidase only. For this reason, glucuronidase was chosen for hydrolysis. In this way, the blank-related background levels could be further reduced to approximately 0.02 µg/l. All in all, the described measures enabled a detection limit of 0.06 µg/l and a quantitation limit of 0.2 µg/l.

12 Discussion of the method

The described analytical method permits the selective and sensitive determination of BHT acid in urine. The rapid and robust analysis allows for the screening of large sample numbers.

By use of the column-switching LC-MS/MS technique, the entire analysis and sample processing (hydrolysis, clean up and quantification) can be performed using only one sample vial, thus minimising process-related analyte losses. The analyte enrichment and the clean-up of the hydrolysed samples is performed on a Waters XBridge C8 column, the analytical separation on a Zorbax RRHD Eclipse Plus C8 column.

Compared to the acidic hydrolysis described in the literature, the enzymatic hydrolysis used in the present method leads to chromatograms with considerably lower noise. The comparative analysis of 22 urine samples from potentially exposed individuals confirmed the suitability of the used enzymatic hydrolysis instead of acidic hydrolysis.

The use of the structurally identical $^{13}C_6$-labelled internal standard renders it possible to compensate for almost all analytical fluctuations, which is confirmed by the good precision data.

In the course of external method verification, calibration was performed using linear regression, with the calibration curve forced through the origin. The working range was also up to 200 µg/l and comparable validation data were obtained.

Instruments used Waters Acquity UPLC coupled with Waters Xevo-TQS tandem mass spectrometer (Waters GmbH, Eschborn, Germany).

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Appendix

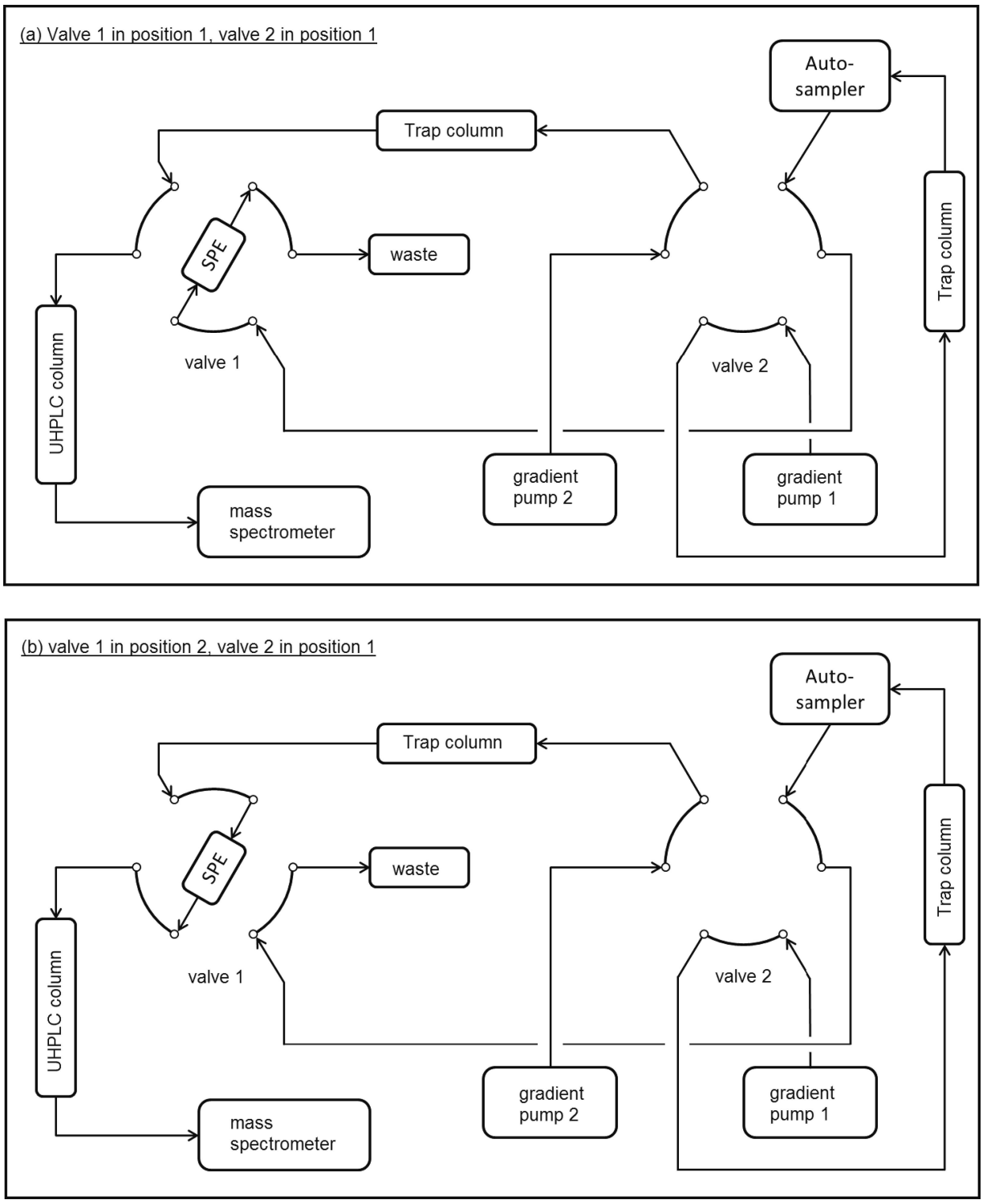

Fig. 2 Schematic representation of the column switching program

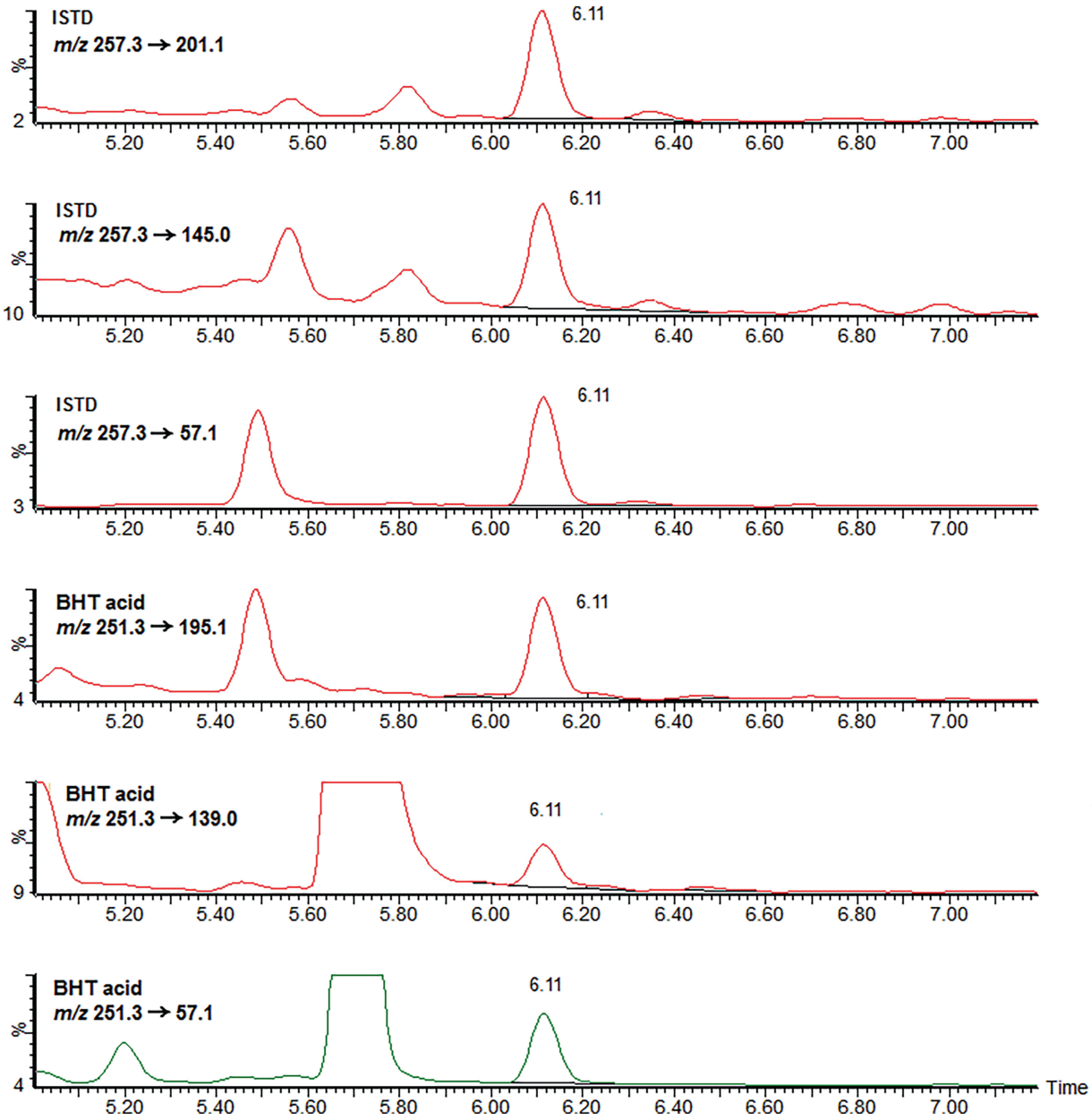

Fig. 3 Chromatogram of a native urine sample with a determined level of BHT acid of approximately 10 µg/l

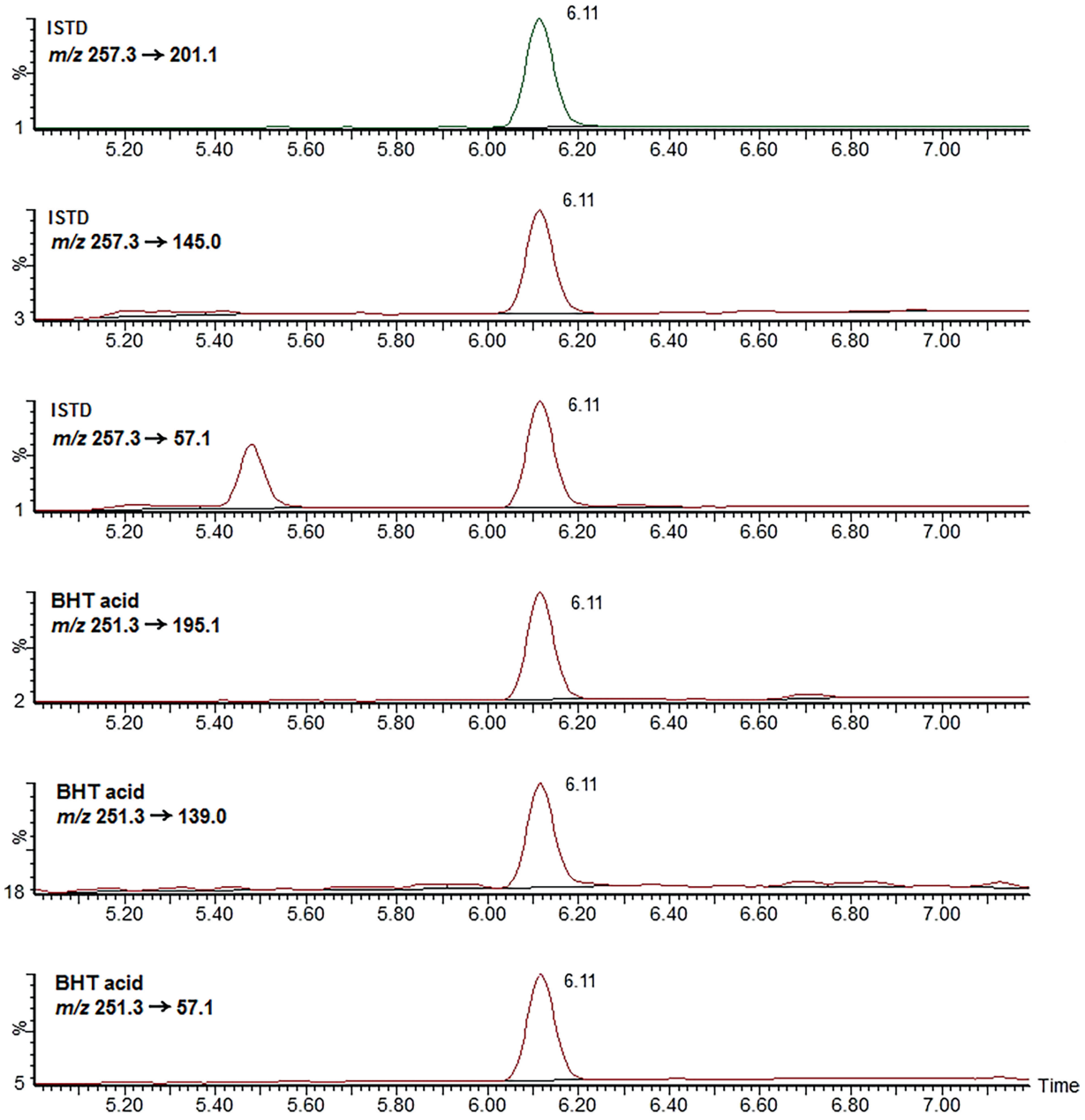

Fig. 4 Chromatogram of a calibration standard in water with a spiked level of BHT acid of 10 µg/l

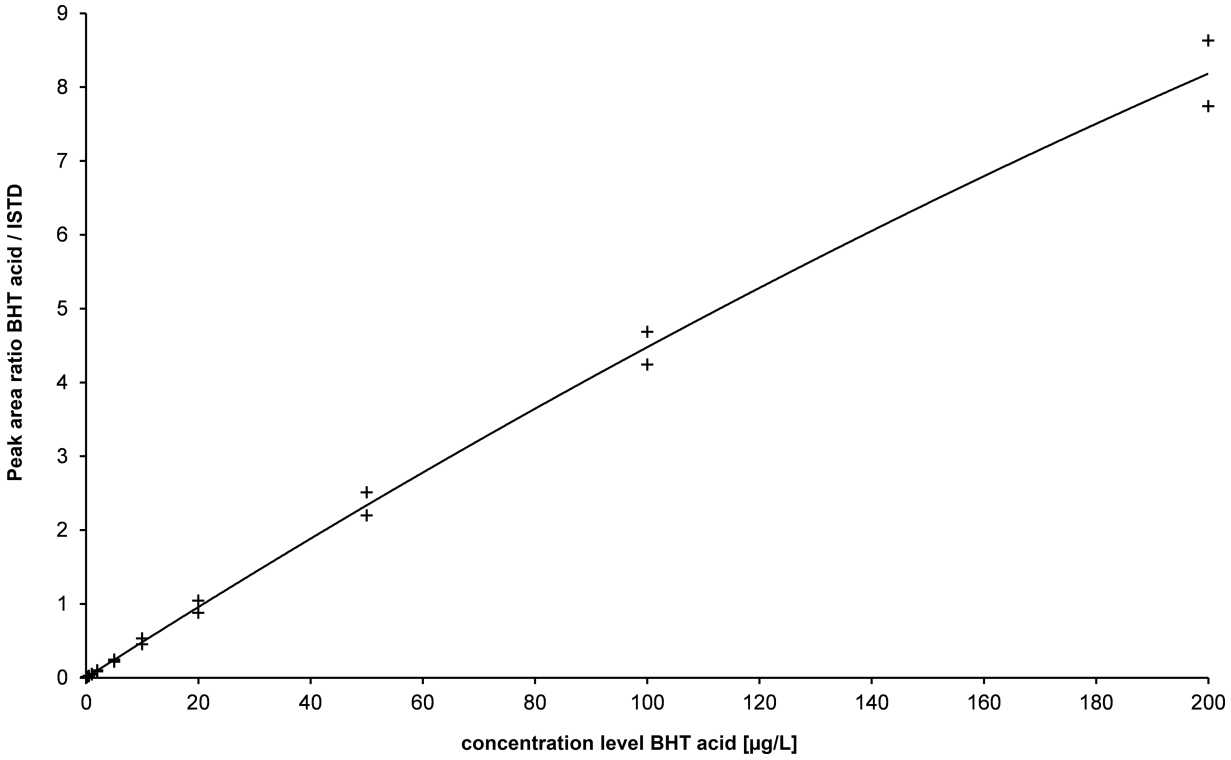

Fig. 5 Calibration curve of BHT acid in water